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Inter-alpha-trypsin inhibitor (ITI): a useful genetic system in paternity testing. Evidence for polymorphic occurrence of *ITI*3* and existence of a new allele, *ITI*4* ★

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Summary. The genetic polymorphism of human inter-alpha-trypsin inhibitor (ITI) has been investigated in sialidase-treated samples by isoelectric focusing on polyarcrylamide gels with a pH range 3.5–9.5 followed by passive blotting with enzyme immunoassay. In 400 blood donors from western Japan, 8 simplified band patterns were observed, 6 of which could be explained by the previously described 3 polymorphic alleles, *ITI*1*, *ITI*2*, and *ITI*3*. The others were products of a new and rare fourth allele designated *ITI*4*, whose expression is also consistent with autosomal codominant inheritance. The frequency of these alleles was 0.440, 0.526, 0.030 and 0.004, respectively. The theoretical exclusion rate for putative fathers in paternity cases was calculated to be 0.228. The ITI system is a useful genetic marker for forensic hemogenetics in Japanese and in Europeans.

Key words: Genetic polymorphism – Inter-alpha-trypsin inhibitor – Japanese population – Paternity tests

Zusammenfassung. Der genetische Polymorphismus des menschlichen Inter-Alpha-Trypsin-Inhibitors (ITI) wurde mit Hilfe der isoelektrischen Fokussierung an Sialidase-behandelten Seren untersucht. Der pH-Bereich war von 3,5 bis 9,5, an die Elektrophorese schloß sich ein passiver Blot an mit anschließendem Enzym-Immuno-Essay. Bei 400 Blutspendern aus West-Japan wurden 8 vereinfachte Bandenmuster beobachtet, von denen 6 durch die bereits früher beschriebenen polymorphen Allele *ITI*1*, *ITI*2* und *ITI*3* erklärt werden konnten. Die anderen Phänotypen waren Produkte eines neuen und seltenen 4. Allels, welches als *ITI*4* bezeichnet wurde und dessen Expression ebenfalls mit der Annahme eines autosomalen kodominanten Erbganges konsistent ist. Die Frequenz dieser Allele war 0,440, 0,526, 0,030 und 0,004. Die theoretische Ausschließungschance für Putativväter in Vaterschaftsfällen wurde mit 0,228 errechnet. Das ITI-System ist ein nützlicher genetischer Marker für die forensische Hämogenetik in der japanischen und europäischen Bevölkerung.

Schlüsselwörter: Genetischer Polymorphismus – Inter-Alpha-Trypsin-Inhibitor – Japanische Bevölkerung – Vaterschaftsanalysen

Introduction

Inter-alpha-trypsin-inhibitor (ITI) is a member of the Kunitz-type protease inhibitor superfamily. It is synthesized in the liver and present in plasma at normal concentrations of 50 mg/ml. ITI is considered to be a single polypeptide chain with a molecular weight of 180 kDa [5, 8, 9]. Recent DNA studies have shown that at least 4 functional genes on 3 different chromosomes encode for 3 heavy chains and one light chain. The resultant proteins are more complex than previously expected and form an ITI family including ITI (225 kDa), pre-alpha-trypsin inhibitor (125 kDa), free HI-30 (30 kDa) and their derivatives. The HI-30 carries a trypsin-inhibitory activity and is included in the proteins of the ITI family [1–4, 6].

The genetic polymorphism of ITI was recently reported by Vogt and Cleve [10] in German and Tyrolian populations using agarose gel isoelectric focusing followed by immunoblotting and immunofixation with polyclonal anti-human ITI sera. It has been suggested that observed phenotypes of ITI are controlled by a single autosomal locus with two common alleles, *ITI*1* and *ITI*2* and one rare allele *ITI*3*. In the present study an improved isoelectric focusing method has been devel-

★ Dedicated to Professor I. Tsuchie on the occasion of his 60th birthday

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oped and ITI system investigated in blood samples from Japanese populations.

Materials and methods

Blood samples. Serum and EDTA-plasma samples for the population study were obtained from 400 unrelated Japanese blood donors living in the Yamaguchi prefecture, the westernmost part of Honshu island. Families were from several prefectures of Japan. The samples were desialylated with an equal volume of sialidase (3 U/ml, pH 5.5; type V, Sigma, St. Louis, MO) at 37°C overnight.

Polyacrylamide gel isoelectric focusing. Isoelectric focusing was carried out with flat-bed gels (200 × 110 × 0.5 mm) prepared as follows: 2 ml acrylamide stock solution containing 29.1% acrylamide and 0.9% BIS, 9.2 ml water, 0.8 ml Ampholine pH 3.5–9.5 were mixed and degassed for 5 min. For polymerization 120 µl ammonium persulfate (30 mg/ml) and 5 µl TEMED were added. The electrode strips contained 1 *M* phosphoric acid as anolyte and 1 *M* sodium hydroxide as catholyte. Separation was carried out at maximal conditions of 30 mA, 15 W and 1000 V for 4 h including 30 min prefocusing. Samples were applied on filter papers (4 × 6 or 3 × 8 mm, Whatman No. 3) and placed for 30 min at the anodic side.

Detection by passive immunoblotting. Transfer of ITI onto an Immobilon membrane (Nihon Millipore, Tokyo) and subsequent immunodetection of band patterns were carried out, as described previously [11], with minor modifications. In brief, an Immobilon membrane was placed on the gel surface with a 1 kg weight for 30 min. After the membrane was quenched with a solution of 0.2% gelatin for 30 min, a 400-fold dilution of rabbit anti-human ITI was added (Dako Japan, Kyoto) for 30 min and subsequently a 500-fold dilution of peroxidase-conjugated swine anti-rabbit immunoglobulin (Dako Japan, Kyoto) for 1 h. The ITI bands were developed in a solution of 3,3-diaminobenzidine and H_2O_2.

Results and discussion

After isoelectric focusing on 5%T and 3%C gels for 4 h, non-treated human hemoglobin A_0 was located as a marker about 3.5 cm from the cathode. The area 1.5–6.5 cm from the cathode was blotted passively for 30 min and ITI bands were developed clearly, as shown in Fig. 1. The immunologically positive reactions were observed in 2 separate zones. The anodal zone near the sample application area included dense and common bands, whereas the cathodal zone near hemoglobin A_0 included sharp and heterogeneous bands. The latter band patterns from 400 Japanese subjects were classified reliably and easily into 8 phenotypes. Five were identical to those resulting from the 3 alleles, *ITI*1*, *ITI*2* and *ITI*3*, as described previously [10]. The homozygous phenotypes consisted of 2 major and a few minor bands, whereas the heterozygous phenotypes showed a composite pattern. Such band patterns were much simpler than those obtained by Vogt and Cleve [10]. This may be due to the difference in the treatment of blood samples with sialidase. In addition, 3 previously unknown phenotypes were encountered in this population sample. One phenotype was composed of 2 major bands located at the same position as the ITI 3 bands, so it appeared to be a homozygous phenotype ITI*3. The others consisted of 4 major bands, two of which corresponded to the ITI 1 or ITI 2 bands

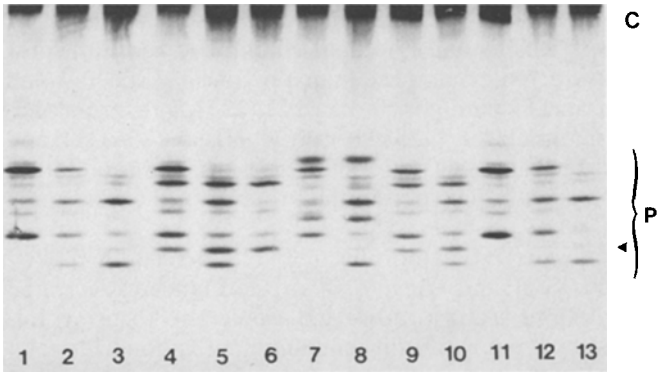

Fig. 1. Immunoblot patterns of ITI phenotypes. Anode at the top. C, P and arrowhead indicate common dense bands, polymorphic zone and location of Hb A_0, respectively. *Lanes 1, 11*: ITI 1; *2, 12*: ITI 2-1; *3, 13*: ITI 2; *4, 9*: ITI 3-1; *5, 10*: ITI 3-2; *6*: ITI 3; *7*: ITI 4-1; *8*: ITI 4-2

Table 1. Inheritance of ITI phenotypes

Matings	*n*	Offspring types							Total
		1	2–1	2	3–1	3–2	3	4–1	
1 × 1	1	1							1
2–1 × 1	7	8	4						12
2–1 × 2–1	7	3	6	3					12
2 × 1	1		2						2
2 × 2–1	11		9	11					20
2 × 2	3			7					7
3–1 × 2	2		1			3			4
3–1 × 3–1	1						1		1
3–2 × 2–1	3		5	1	1	1			8
3–2 × 2	1					2			2
4–1 × 1	1							2	2
Total	38	12	27	22	1	6	1	2	71

Table 2. Distribution of ITI phenotypes and allele frequencies in 400 Japanese

Phenotypes	Number		χ^2
	Observed	Expected	
ITI 1	80	77.44	0.0846
ITI 2–1	184	185.24	0.0083
ITI 2	110	110.78	0.0055
ITI 3–1	7	10.56	1.2002
ITI 3–2	15	12.63	0.4447
ITI 3	1	0.36	
ITI 4–1	1	1.32	
ITI 4–2	2	1.58	0.1219
ITI 4–3	0	0.09	
ITI 4	0	0.01	
Total	400	400.01	1.8652

Allele frequencies: *ITI*1* = 0.4400, *ITI*2* = 0.5263, *ITI*3* = 0.0300, *ITI*4* = 0.0038

$\chi^2 = 1.8652$, $df = 2$, $0.50 > P > 0.30$

Table 3. Comparison of ITI allele frequencies and theoretical exclusion probabilities (P) in different populations

Populations	*n*	ITI allele frequencies				P (%)	References
		*ITI*1*	*ITI*2*	*ITI*3*	*ITI*4*		
German	248	0.5746	0.4173	0.0081	–	19.5	[10]
Tyrolian	124	0.5766	0.4234	–	–	18.5	[10]
Japanese	400	0.4400	0.5263	0.0300	0.0038	22.8	this study

and two were anodal to the ITI 1 bands. These phenotypes were heterozygous, carrying a new allele designated *ITI*4* in combination with the common *ITI*1* or *ITI*2* alleles. Recently the ITI 4-2 phenotype has also been identified in a Korean (H. Cleve, personal commun.).

In order to confirm the genetic basis of ITI phenotypes, family studies including 38 matings with 71 offsprings were performed (Table 1). No unexpected types were found in the children, supporting the hypothesis that ITI is controlled by autosomal codominant Mendelian inheritance at a single locus. The new allele, *ITI*4*, was also confirmed to segregate in a similar fashion. The gene for the light chain of ITI (HI-30) is assigned to chromosome 9q32–33 [1] and seems to be linked to the polymorphic markers ABO, AK1, ALAD and ORM systems, which are located at 9q34.1–34.2, 9q34.1–34.2, 9q34, and 9q31–32, respectively [7, 12]. Association analysis between ABO phenotypes and ITI alleles was performed in 2×2 table by χ^2 statistics. No evidence for a linkage disequilibrium between ABO blood groups and ITI was obtained.

Table 2 summarizes the results of the population study for ITI in 400 unrelated Japanese subjects. The allele frequencies were estimated to be 0.440, 0.526, 0.030 and 0.004 for *ITI*1*, *ITI*2*, *ITI*3* and *ITI*4*, respectively. The observed distribution was in good agreement with the Hardy-Weinberg law.

Table 3 shows the differences in the distribution of ITI allele frequencies between European and Japanese populations together with the values of the theoretical exclusion probability for paternity testing. *ITI*1* is predominant over *ITI*2* in the Europeans, but the reverse is found in Japanese. The most striking feature is the polymorphic occurrence of *ITI*3* in Japanese with an allele frequency of 0.03. This allele is present in Germans at a rare frequency of 0.008, but is not found in Tyrolians. ITI may be a potential system to distinguish between Europeans and Asians and *ITI*3* may be a potential marker to characterize Asians. The single exclusion chance of ITI was calculated to be 0.195, 0.185 and 0.228 in German, Tyrolian and Japanese populations, respectively. The value in Japanese is higher than that in Europeans and is comparable to that found in the MNSs system.

Commercially available polyclonal anti-human ITI sera contain antibodies against one light chain and 2 heavy chains of ITI [3] and may detect all or some proteins of the ITI family. The ITI polymorphism has not yet been assigned to a specific protein or polypeptide. Nevertheless, the ITI system is a useful genetic marker in paternity tests.

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